ARTICLE NO. VY978709

A Single Sequence Change Destabilizes the Influenza Virus Neuraminidase Tetramer

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Received April 21, 1997; returned to author for revision June 2, 1997; accepted June 25, 1997

A single change (E119G) in the influenza A virus N9 neuraminidase (NA) results in resistance of the enzyme to the NA inhibitor 4-Guanidino-Neu5Ac2en (4-GuDANA). This change causes a salt link between Glu119, which sits in a pocket in the bottom of the active site of the enzyme, and the 4-guanidinium moiety of the inhibitor to be lost. NA "heads" of the resistant enzyme produced only a few small crystals under conditions in which the wild-type enzyme readily formed large crystals. These small crystals were of sufficient quality to yield X-ray crystallographic data which confirmed the E119G change and demonstrated the presence of electron density representing either a strong structural–water molecule or an anionic species in place of the glutamate carboxylate. NA heads of the resistant enzyme also have greatly reduced NA activity per milligram of total protein. We have now found that the mutant NA heads consist predominantly of monomers with a few dimers and tetramers, as determined by electron microscopic analysis of the protein. The low level of enzymatic activity as well as the small number of crystals obtained were probably from the few tetramers remaining intact in the preparation. The purified wild-type and 4-GuDANA-resistant enzymes were treated with the homobifunctional NHS-ester cross linker, DTSSP. SDS–PAGE analysis of the treated enzymes clearly revealed cross-linked dimers of the wild-type enzyme. In contrast, only a small proportion of the 4-GuDANA-resistant neuraminidase was cross-linked. An examination of the known X-ray crystallographic structure of the wild-type NA reveals a salt bridge between Glu119 and Arg156 of the same monomer. Arg156 is a conserved amino acid that is situated at the interface between monomers, and a salt link between this amino acid and Glu119 may contribute to the stability of enzyme tetramers. It is suggested that the E119G alteration in the 4-GuDANA-resistant NA leads to the abrogation of this interaction and thus to the instability of the NA tetramers.

INTRODUCTION

Influenza virus neuraminidase (one of two glycoprotein antigens on the surface of influenza virus particles — the other being the hemagglutinin) is a homotetrameric molecule consisting of a square box-like head on the top of a long stalk which attaches the molecule to the lipid bilayer of the enveloped virus particle (Laver and Valentine, 1969; Wrigley *et al.,* 1973; Colman and Ward, 1985). The function of the neuraminidase is to remove sialic acid residues from the surface of infected host cells and from the hemagglutinin and neuraminidase of newly formed virus particles in order to facilitate release of the progeny virus and allow it to spread within the body (reviewed by Murphy and Webster, 1990).

The X-ray crystal structures of influenza A N2, N6, N8, and N9 neuraminidases, as well as influenza B virus neuraminidase, have been determined (Varghese *et al.,* 1983; Varghese and Colman, 1991; Tulip *et al.,* 1991; Burmeister *et al.,* 1992; Taylor *et al.,* 1993) and, despite large differences in the primary amino acid sequences, the overall topology of the tetramer is more or less conserved and the structure of the catalytic site is absolutely conserved (Burmeister *et al.,* 1992; Bossart-Whitaker *et al.,* 1993). The fact that the catalytic site of the neuraminidase of all influenza viruses has the same structure stimulated the development of inhibitors of this enzyme as anti-viral agents for influenza, since such an inhibitor would be effective against all influenza viruses, even those which have not yet appeared in humans.

One such inhibitor, 4-guanidino-Neu5Ac2en or GG-167 (hereafter referred to as 4-GuDANA), has been described (von Itzstein *et al.,* 1993) and is currently undergoing clinical evaluation (Hayden *et al.,* 1996). Mutant influenza viruses which are resistant to GG-167 have been selected in cell culture (Staschke *et al.,* 1995; Blick *et al.,* 1995; Gubareva *et al.,* 1996) and in some of these the neuraminidase had a single amino acid alteration of Glu to Gly or Glu to Ala at position 119 (N2 numbering). There is a strong salt link between the carboxylate of Glu 119 and the C-4 guanidinium group of GG-167 (Varghese *et al.,* 1995), and the amino acid change at position 119 eliminates this interaction, thus reducing the enzyme inhibitory action and antiviral activity of 4-GuDANA.

In this report, we provide X-ray crystallographic evi-

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0042-6822/97 $25.00

dence which corroborates the E119G alteration in the 4-GuDANA-resistant N9 neuraminidase (NA) reported previously (Staschke *et al.,* 1995). We also reported previously that the mutant NA (E119G) had greatly reduced specific activity, compared to wild-type NA, for fetuin and 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUN) substrates (Staschke *et al.,* 1995). It was not known if this loss of enzyme activity was caused by a change in the catalytic activity of the mutant NA or whether the NA had become denatured in some way, leading to a loss of activity. In an elegant study, McKimm-Breschkin *et al.* (1996) have shown that the specific activity of the small amount of residual active neuraminidase in preparations of E119G mutant N9 NA "heads" is the same as that of wild-type N9 NA. Therefore, the mutation E119G had not altered the catalytic properties of the NA, but had made it unstable in some way.

Using electron microscopic analysis we have now demonstrated that, in contrast to preparations of wild-type neuraminidase heads, which consist of virtually 100% tetramers, preparations of the E119G mutant N9 neuraminidase heads consist predominantly of monomers with some dimers and only a few tetramers detectable. Cross-linking experiments showed that wild-type enzyme monomers were readily cross-linked to form dimers, whereas monomers of 4-GuDANA-resistant enzyme monomers were poorly cross-linked into dimers.

MATERIALS AND METHODS

Preparation and purification of neuraminidase heads

Purified neuraminidase heads were obtained as previously described (Laver, 1969; Laver *et al.,* 1984). Briefly, virus particles were obtained from the allantois of 11-day embryonated chicken eggs infected with wild-type or 4-GuDANA-resistant influenza A/NWS/33_{HA}-A/Tern/Australia/G70c/75_{NA} (H1N9; hereafter referred to as A/NWS-G70c), a reassortant virus which contains an N9 neuraminidase (Laver *et al.,* 1984). The virus was purified by adsorption–elution on chicken red blood cells followed by sucrose gradient centrifugation. The virus particles were suspended in 0.15 *M* NaCl, 0.1 *M* sodium phosphate, pH 7.0, and incubated with Pronase (Calbiochem) for 2 days at 20°. Virus cores were removed by centrifugation at 30,000 rpm for 1 hr and the supernatant, which contained 100% of the neuraminidase activity, was centrifuged at 50,000 rpm for 20 hr using a Ti 60 rotor. The neuraminidase heads formed a pellet which had a crystalline appearance. This pellet was dissolved in saline (0.5 ml) and the neuraminidase heads were purified by sucrose density gradient centrifugation (5–20% sucrose in 0.15 *M* NaCl) for 8 hr using a SW 65 rotor at 58,000 rpm, 5°. Fractions containing neuraminidase activity were pooled and dialyzed against saline and then against 70% saturated $(NH_4)_2SO_4$. The precipitated neuraminidase heads were centrifuged, dissolved in a minimum volume of saline, and dialyzed against saline.

Crystallization, X-ray data collection, processing, and model refinement

The 4-GuDANA-resistant enzyme mutant was crystallized from hanging drops by vapor diffusion at 20° over a mixture of 1.4 *M* KH_2PO_4 and 3 *M* K_2HPO_4 in the ratio of 8:3 (v/v). They belong to cubic space group I432 with unit cell parameter a = 181.08 Å, having one molecule per asymmetric unit. These crystals have a similar morphology but are significantly smaller (approximately 0.2 mm in the largest dimension) than wild-type crystals when grown under similar conditions (Fig. 1). A crystal was sequentially soaked in increasing concentrations of glycerol cyroprotective agent up to 40% (v/v with mother liquor) over a total soaking time of 5 min. It was then suspended in the cryoprotected liquor across a mohair loop and flash-cooled to a temperature of 100 K for X-ray data collection on beam line 7.2 at the synchrotron radiation source (SRS; Daresbury, UK). Data were collected on an 18-cm-diameter MAR Research image plate placed at a distance of 119 mm from the crystal, giving a maximum resolution of 2.07 Å at the edge of the detector for the incident X-ray wavelength of 1.488 Å. An oscillation angle of 0.6°/image was used, and a total of 22.8° of data were collected, all from one crystal. The diffraction data were reduced with the DENZO program (Minor, 1993) and intensities were scaled with SCALEPACK (Otwinowski, 1993). A total of 83,843 observations were measured, yielding 29,944 unique reflections with an R_{merge} of 6.2%, representing 96.6% of all that are theoretically possible for resolution range 19.0–2.07 Å. Only fully recorded reflections were included in the scaled data set. The highest resolution shell (2.2 to 2.07 Å) has a completeness of 97.5% with an R_{merge} value of 14.2%. For the molecular replacement search model, we used the enzyme coordinates from the N9 NA/4–GuDANA complex (Varghese *et al.,* 1995; Protein Data Bank entry 1NNC; resolution 1.8 Å, *R* value = 15.6%) rather than the coordinates from native N9 NA (PDB entry code 7NN9; resolution 2.0 Å, *R* value = 15.2%) because of the higher resolution of the experimental data of the former. The resolution, quality, and completeness of our experimental data were sufficient to avoid model bias from being introduced by the starting model. Subsequently, the structure of the 4-GuDANA-resistant N9 NA, as described here, was compared to the available structure of native N9 NA (reference 7NN9; resolution 2.0 Å, *R* value = 15.2%). The space group and unit cell parameters of the mutant NA crystal were the same as for the wild-type N9 crystal. After rigid body refinement of the starting N9 model (with the 4-GuDANA removed) against the mutant data, conventional refinement of positional and thermal parameters was performed using the X-PLOR program (Brünger,

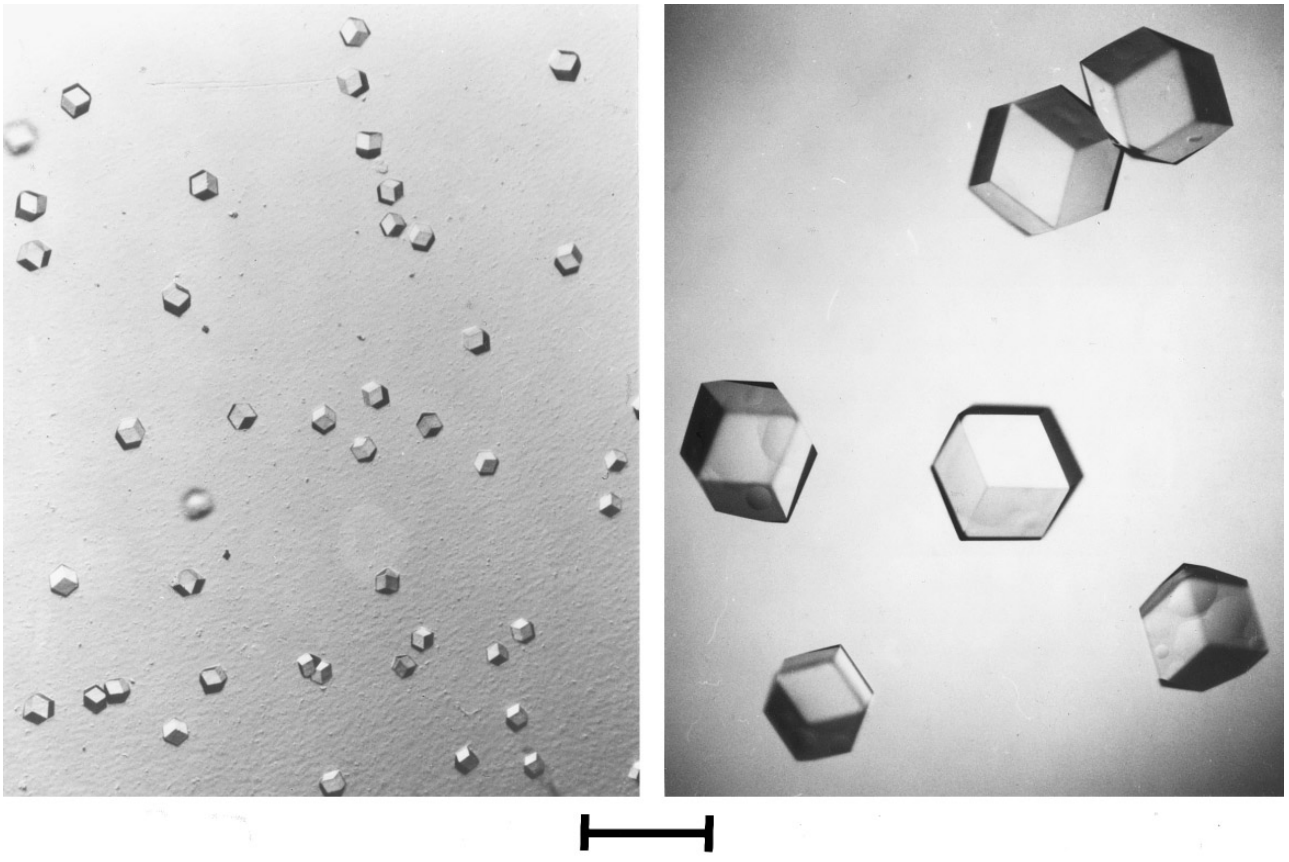


FIG. 1. Photographs of wild-type N9 neuraminidase crystals (right) and E119G (4-GuDANA-resistant) N9 neuraminidase crystals (left). Crystals of both enzymes were grown in hanging drops by vapor diffusion over potassium phosphate under the same conditions as described under Materials and Methods. Crystals of the mutant enzyme formed but no crystals larger than 0.2 mm were obtained. Bar, 1.0 mm.

1992). Visual inspection and manual correction of the model between rounds of refinement were carried out using the QUANTA interactive graphics program (Molecular Simulations, 1994). A final crystallographic R value of 20.1% for 26,711 reflections with $F > 2\sigma$ in the resolution range 6.0–2.07 Å was obtained. An R_{free} value (Brünger, 1992) of 24.4% was calculated using a random selection of approximately 10% of the reflections which were omitted from refinement. The RMS deviation from ideal bond lengths and bond angles was 0.019 Å and 1.3°, respectively. A Ramachandran (ϕ, ψ) plot clearly indicated that the structure does not have any violations (data not shown). A mean positional error of approximately 0.2 Å was estimated from a Luzzati plot (Luzzati, 1952). Summaries of the crystallographic data and structure statistics are given in Tables 1 and 2. The coordinates of the X-ray structure of the 4-GuDANA-resistant influenza A N9 NA will be submitted to the Protein Data Bank (Brookhaven National Laboratories).

Enzyme kinetic analysis and substrate preference

Enzyme kinetic analyses of purified 4-GuDANA-resistant or wild-type N9 neuraminidase were conducted using MUN as the substrate in a standard fluorometric assay as previously described (Myers *et al.,* 1980). To determine the substrate preference of the 4-GuDANA-resistant and wild-type enzymes, neuraminidase activity was assayed using the thiobarbituric acid (TBA) assay as

TABLE 1

X-Ray Crystallographic Data Summary

Space group	I432
Unit cell parameters, Å	
a, Å	181.08
Resolution range, Å	19.0–2.07
Number of observations	83,843
Number of unique reflections	29,944
Average redundancy	2.8
Completeness, %	
Overall	96.9
Outershell (2.07–2.2 Å)	97.5
R_{merge},[a] %	
Overall	6.2
Outershell (2.07–2.2 Å)	14.2
Average I/σ(I)	
Overall	11.5
Outershell (2.07–2.2 Å)	3.8

[a] $R_{merge} = \Sigma | I_i - \langle I_i \rangle | / \Sigma I_i$, where I_i is the intensity of an individual measurement and $\langle I_i \rangle$ is the mean intensity of this reflection.

TABLE 2

Model Refinement Statistics

Number of atoms in model	
Overall	3325
Protein	3062
Carbohydrate	134
Ca^{2+}	1
Water molecules	136
Mean temperature factor, Å^2	14.8
Distance restraint information, Å	
Bond distance	0.019
Angle distance	0.037
Planar 1–4 distance	0.046
Deviation from ideal bond angles, degrees	1.3
Planar groups, Å	0.023
Chiral center volumes, Å^3	0.194
Single-torsion contact, Å	0.196
Multiple-torsion contact, Å	0.238
Torsion angles, degrees	
Planar	3.4
Staggered	16.1
Orthonormal	28.9
R_{free}, %[a]	24.4
Crystallographic R value,[b] %	20.1

[a] $R_{free} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$ has been calculated from 10% of the reflections removed from the refinement.

[b] R value $= \Sigma||F_o| - |F_c||/\Sigma|F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

previously described with modifications (Schauer, 1978). Initially, the activity of each enzyme was confirmed and standardized using fetuin as a substrate (data not shown). The substrates, 3′-*N*-acetylneuramin-lactose sodium salt or 6′-*N*-acetylneuramin-lactose sodium salt (Sigma Chemical Co., St. Louis, MO), were resuspended in Dulbecco's phosphate-buffered saline and diluted with D-PBS to 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078 m*M*. Final concentration of the substrate was further diluted 1:2 by the addition of enzyme. For the neuraminidase reactions, 50 μl of each substrate concentration was mixed with 50 μl of either wild-type or 4-GuDANA-resistant influenza A/NWS-G70c N9 neuraminidase. The reactions were incubated at 37° for 3 hr and then processed for TBA color development as previously described (Schauer, 1978). The absorbance at 540 nm was quantified using a Vmax Kinetic Microplate Reader (Molecular Devices).

Electron microscopic analysis of neuraminidase heads

Both wild-type and 4-GuDANA-resistant neuraminidase heads were purified as described above and examined by electron microscopy. The preparations of enzyme heads were absorbed to carbon-coated electron microscope grids and stained negatively with 1% uranyl acetate before electron microscopic examination as described previously (Els *et al.,* 1985).

Cross-linking of purified neuraminidase heads

Purified 4-GuDANA-resistant or wild-type N9 neuraminidase was cross-linked using the water soluble homobifunctional NHS-ester cross linker, 3,3′-dithiobis (sulfosuccinimidylproprionate) (DTSSP; Pierce Chemical Co., Rockford, IL). The enzyme was incubated at room temperature for 45 min in a total volume of 20 μl of PBS containing 0 (mock cross-linked) or 1 m*M* DTSSP. After cross-linking, the enzyme was boiled in sample buffer containing sodium dodecyl sulfate without β-mercaptoethanol to avoid reducing the dithio group of the cross-linking reagent and electrophoresed through a 10–20% polyacrylamide gel. Protein bands were visualized by Coomassie blue staining and sizes were deduced by comparison to protein standard markers.

CHARMM force field calculations

All force field calculations were performed with the CHARMM (Chemistry at Harvard Molecular Mechanics) program (Brooks *et al.,* 1983; Molecular Simulations, 1994a) on a Silicon Graphics workstation. Hydrogen atom positions were built into the X-ray structures using the HBUILD algorithm (Brünger and Karplus, 1988). Subsequently, all nonhydrogen atom positions were fixed and the hydrogen atom positions were optimized to zero energy gradient using the adopted basis Newton–Raphson algorithm (Brooks *et al.,* 1983). Calculations were performed with a constant dielectric equal to 80 in order to simulate the enzymes in aqueous solution. The interaction energies obtained are enthalpies and do not include entropy effects.

RESULTS

Substrate specificity and enzyme kinetic analysis of 4-GuDANA-resistant and wild-type N9 neuraminidase

The action of the wild-type or 4-GuDANA-resistant N9 neuraminidase on two different substrates was evaluated. Both enzymes demonstrated a preference for the 2-3′ linked rather than the 2-6′ linked sialic acid substrate (Fig. 2). Using purified neuraminidase heads isolated by pronase digestion of egg-grown virus, enzyme kinetic analysis demonstrated that wild-type and 4-GuDANA-resistant neuraminidases displayed similar K_m values for the fluorescent substrate MUN but the resistant enzyme had a sevenfold higher K_i for 4-GuDANA (Table 3). The wild-type enzyme displayed a V_{max} of 82 μmol/min/mg protein and a K_{cat} of 3762 μmol product/μmol enzyme/min. Accurate determinations of V_{max} and K_{cat} could not be obtained for the resistant neuraminidase since the amount of active enzyme in the total protein preparation could not be accurately determined due to degradation and instability of the enzyme tetramer (see below).

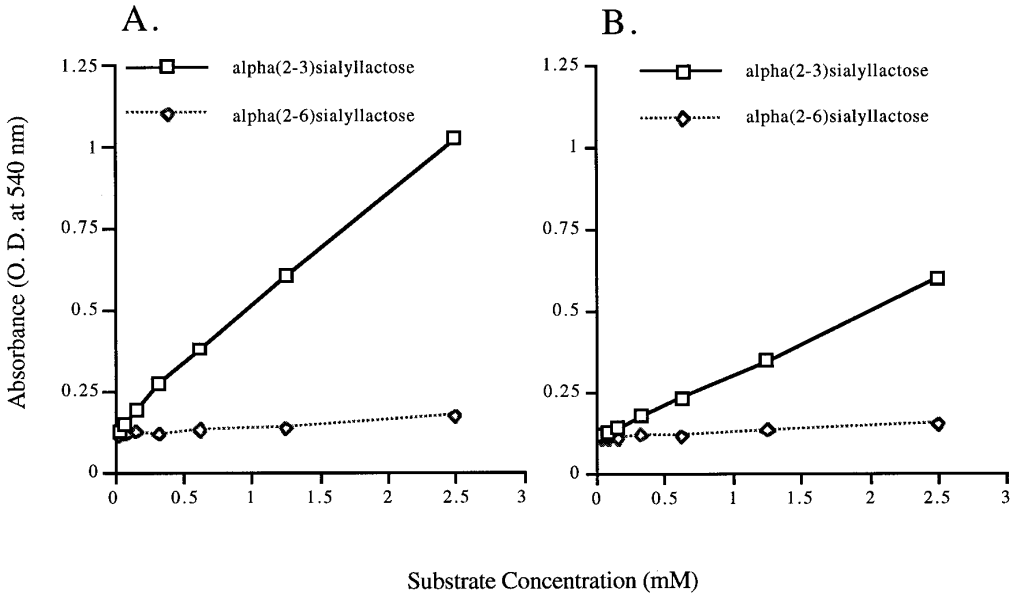


FIG. 2. Cleavage specificity of wild-type and 4-GuDANA-resistant N9 neuraminidases. Neuraminidase activity was evaluated using the thiobarbituric assay with either 3′-*N*-acetylneuramin-lactose sodium salt [α(2-3)sialyllactose] or 6′-*N*-acetylneuramin-lactose sodium salt [α(2-6)sialyllactose] as the substrate. (A) Wild-type N9 neuraminidase; (B) 4-GuDANA-resistant N9 neuraminidase.

Electron microscopic analysis of 4-GuDANA-resistant N9 neuraminidase heads

As shown in Fig. 3A, electron microscopic analysis of wild-type N9 neuraminidase heads reveals the characteristic tetrameric structure of the active enzyme. This sample consisted of a homogeneous population of tetrameric neuraminidase heads in various orientations. In contrast, the preparation of 4-GuDANA-resistant neuraminidase heads contained very few dimers and tetramers (less than 10%), with the predominant population consisting of monomers (Fig. 3B). These results indicate that the single mutation of 119 Glu to Gly in the neuraminidase dramatically destabilizes the enzyme tetramers, causing them to dissociate into monomers.

Cross-linking studies

4-GuDANA-resistant (4-GuDANA^r) or wild-type neuraminidase was incubated with 0 (−) or 1 m*M* (+) DTSSP, boiled in sample buffer without β-mercaptoethanol, and analyzed by SDS–polyacrylamide (10–20%) gel electrophoresis. As shown in Fig. 4, cross-linking was observed readily for the wild-type neuraminidase (~97-kDa band). Only a faint band at this position can be seen for the 4-GuDANA-resistant enzyme incubated with DTSSP. Additionally, a more rapidly migrating band can be seen in the resistant enzyme preparation, most likely as a result of protein degradation. These results indicate that, in solution, the mutant N9 enzyme consists mostly of monomers.

TABLE 3

Enzyme Kinetic Analysis of 4-GuDANA-Resistant and Wild-type N9 Neuraminidases

	Wild-type	Mutant	Significance
K_m (μM)	67.7	75	NS
K_i (n*M*)	48.1	328.5	$P < 0.005$

Crystal structure of the mutant 4-GuDANA-resistant N9 neuraminidase

Overall, the structure of the 4-GuDANA-resistant neuraminidase is similar (RMS fit of the alpha carbon atoms = 0.35 Å over all 388 residues) to that of the native wild-type N9 neuraminidase (reference 7NN9) except for a few side chain positions, including amino acid residues Arg 82, Lys261, Lys273, Arg304, Arg414, and Arg465, each of which has a different spatial orientation. Also the structural water pattern was slightly changed. A strong continuous electron density, a piece of which extended from the oligosaccharide attached at Asn86, was interpreted as a second *N*-acetylglucosamine and included in further refinement. The absence of electron density around the amino acid residue at position 119 clearly indicates the presence of glycine instead of glutamic acid (E119G) (Fig. 5). A strong electron density peak was found occupying the position of the side chain of the former carboxyl group of Glu119 (Peak 1). Based on the shape of the electron density, this peak might be interpreted as a strong structural water molecule (Blick *et al.,* 1995). Another 5σ cigar-shaped electron density peak was found in the position normally occupied by the carboxylate group of sialic acid in the enzyme–substrate

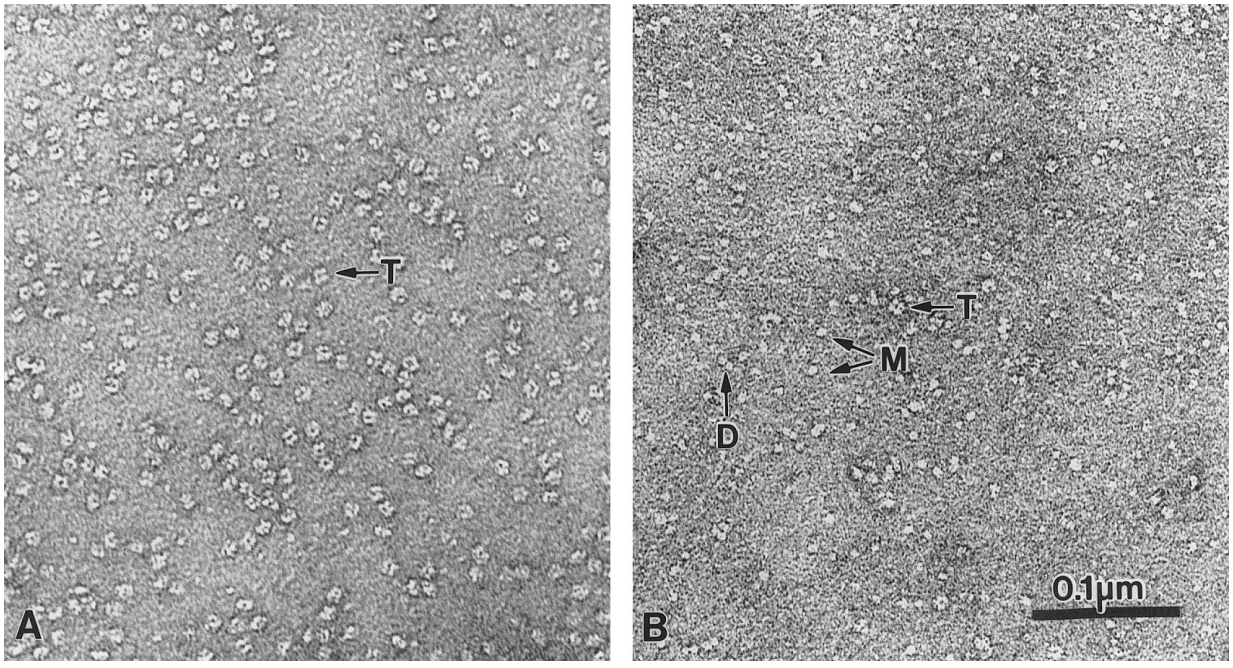


FIG. 3. Electron micrographs of negatively stained neuraminidase "heads" from (A) wild-type influenza A/NWS-G70c virus and (B) 4-GuDANA-resistant influenza A/NWS-G70c virus with the single alteration of Glu 119 to Gly. T, tetramers; D, dimers; M, monomers.

complex (1NNC) (Peak 2; Fig 5). Blick *et al.* (1995) have interpreted this as three noninteracting water molecules. Our data reveal a peak in a similar position, although this peak is not large enough to accommodate three noninteracting water molecules. Taking into consideration the fact that this density is surrounded by three positively charged amino acids (Arg118, Arg292, and Arg374), it could be interpreted to represent an anionic molecule. Also noted in the active site region is an electron density peak between Arg152 and Arg 225 which represents a phosphate group, most likely originating from the mother liquor.

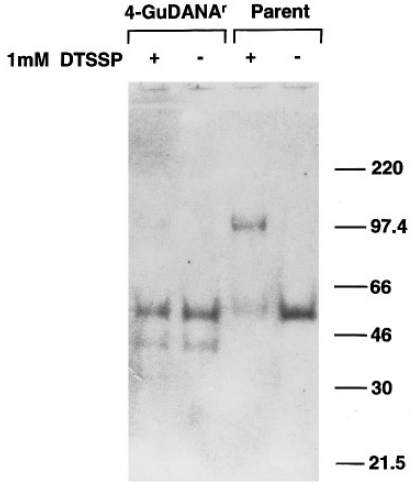


FIG. 4. Cross-linking of 4-GuDANA and wild-type N9 neuraminidase. Purified 4-GuDANA-resistant (4-GuDANA^r) or wild-type neuraminidase was incubated with 0 (−) or 1 m*M* (+) DTSSP, boiled in sample buffer without β-mercaptoethanol, and analyzed by 10–20% gradient polyacrylamide gel electrophoresis. Cross-linking was observed readily for the wild-type neuraminidase (97-kDa band). A faint 97-kDa band can be seen for the 4-GuDANA-resistant enzyme.

Results of CHARMM analysis

CHARMM force field calculations (Brooks *et al.,* 1983) were performed on the wild-type N9 neuraminidase and on the 4-GuDANA-resistant (E119G) enzyme in order to investigate the interaction energies of association of the monomers into a hypothetical tetramer. The results of the calculations indicated that there is a more negative interaction energy for the formation of the tetramer for the wild-type enzyme than there is for the E119G mutant enzyme. The difference in interaction energies is −71.2 kcal/mol. In our calculations, we also focused on the interaction of Arg156, located at the intersubunit interface (Fig. 6), with Glu119 and the surrounding enzyme environment in order to investigate the role of this interaction in tetramer formation and stability. The results of these calculations indicate that Arg156 has a −3.1 kcal/mol favorable energy of interaction with the surrounding enzyme environment in the wild-type enzyme. In contrast, Arg156 in the mutant enzyme has a 17.7 kcal/mol unfavorable energy of interaction with the surrounding environment. In the wild-type enzyme, Glu119 is close enough (∼3.0 Å) to Arg156 for the formation of a salt link between the carboxylate of the glutamate and the guanidino of the arginine. Furthermore, the charge interactions of Arg 156 are stabilized by Glu119, while in the mutant enzyme, there is greater destabilization associ-

ated with Arg156. The general trend of these force field calculations is in accord with experimental results and indicates that the interaction between Arg156 and Glu119 plays a dramatic role in neuraminidase tetramer formation and stability.

DISCUSSION

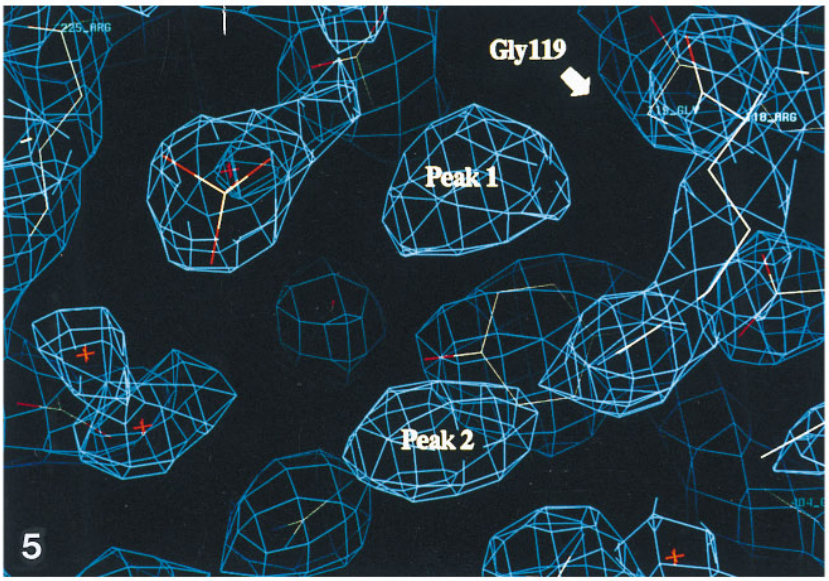
Resistance to the potent and selective influenza neuraminidase inhibitor 4-GuDANA is, in some cases, due to a sequence change in the neuraminidase of Glu 119 to Gly or Ala (Staschke *et al.,* 1995; Blick *et al.,* 1995; Gubareva *et al.,* 1996). This alteration eliminates a salt bridge between the carboxylate of Glu119 and the C-4 guanidinium moiety of the inhibitor (von Itzstein *et al.,* 1993; Varghese *et al.,* 1995), reducing its binding affinity and hence its inhibitory activity. In this report, we obtained crystals of the 4-GuDANA-resistant N9 neuraminidase, the gene of which was originally shown by sequence analysis to contain a single nucleotide change leading to the alteration of Glu119 to Gly (Staschke *et al.,* 1995). X-ray diffraction data confirm the absence of a Glu119 and its replacement with Gly. Additionally, electron density mapping revealed the presence of what might be a strong structural water molecule which takes the place of the Glu119 carboxylate. Blick *et al.* (1995) have also noted the presence of this electron density, which they interpreted to be a water molecule which can interact with the C-4 guanidinium moiety of 4-GuDANA in a manner similar to that in which the carboxylate of Glu119 in the wild-type enzyme interacts with this group of the inhibitor. However, we did not detect any hydrogen bonds between the putative water molecule and amino acid side chains in this region. Additionally, the height and shape of the electron density cloud can accommodate more than a single water molecule. Taking these observations into consideration, we cannot rule out the possibility that this peak of electron density represents an anionic species, possibly a constituent of the buffer system used in the purification of the enzyme.

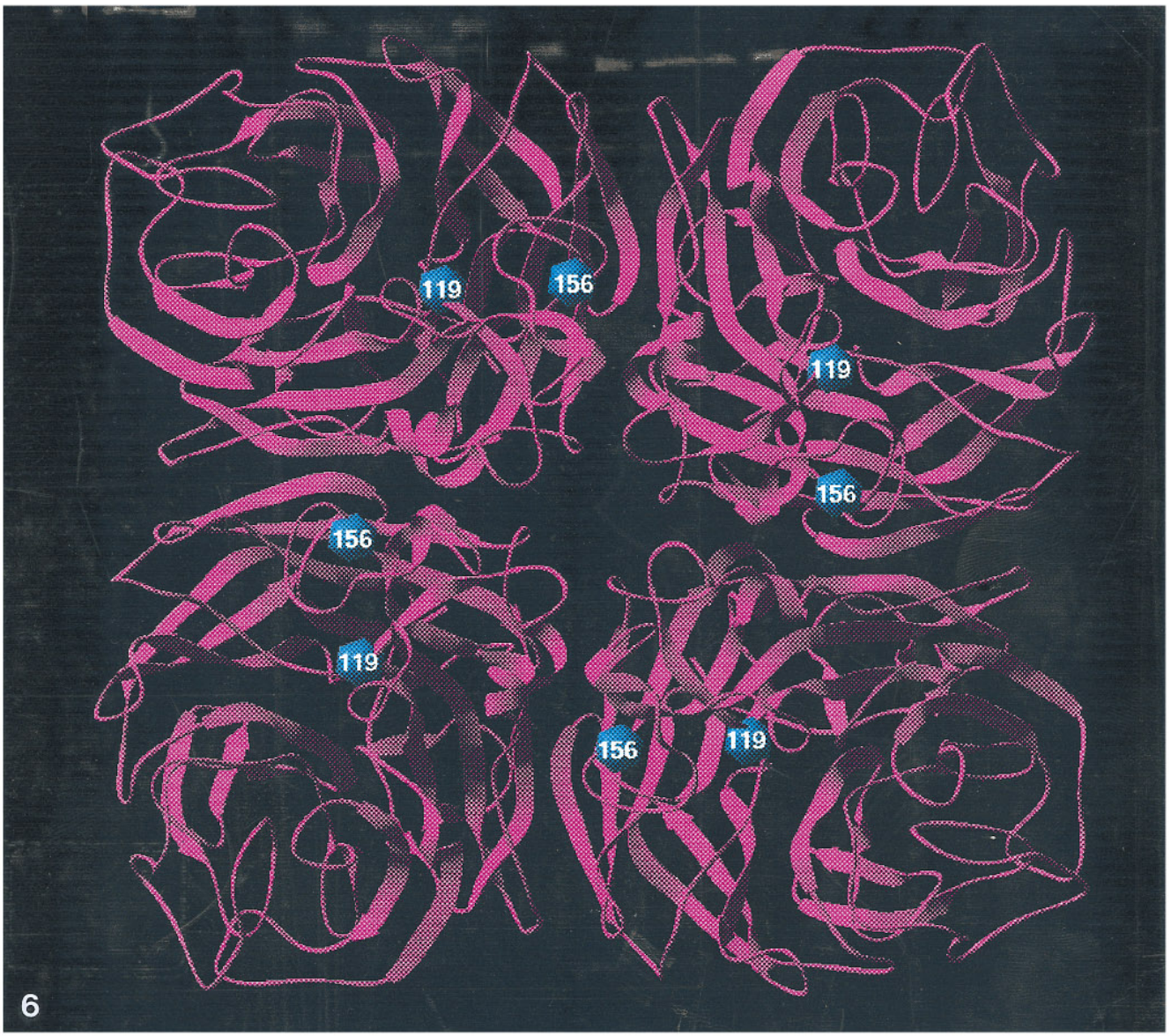
We reported previously (Staschke *et al.,* 1995) that purified, pronase released neuraminidase heads having the mutation E119G (which resulted in the resistance to 4-GuDANA) had approximately 5% of the neuraminidase activity for the fetuin substrate as wild-type N9 NA heads prepared and purified in the same way. This value was obtained by measuring the neuraminidase activity of the purified enzyme preparations and relating this to total protein content, as assayed by standard chemical methods. This apparently low specific activity of the mutant enzyme could be explained by one of two possibilities: (1) the purified mutant enzyme polypeptide was correctly folded into its native enzymatically active state but the E119G mutation had reduced its turnover rate for fetuin and MUN substrates to 5% that of the wild-type enzyme; or (2) the mutant enzyme had the same turnover rate for fetuin and MUN substrates but was mostly denatured so that only 5% was correctly folded into an enzymatically active state. By measuring the specific activity of residual native neuraminidase in preparations of E119G mutant N9 neuraminidase heads, McKimm-Breschkin *et al.* (1996) have shown that the second possibility is the correct explanation for the low level of enzyme activity in preparations of the 4-GuDANA-resistant (E119G) mutant N9 neuraminidase. The amount of native, correctly folded enzyme was measured in an ELISA using a monoclonal antibody (NC10) which bound to native N9 neuraminidase, but not to the denatured (unfolded) neuraminidase. It was shown that when NWS-G70c virus with the E119 mutant neuraminidase was stored at 37° for several days, the amount of native enzyme fell dramatically, whereas that of the wild-type virus remained constant. In these tests the virus concentration was adjusted so that the neuraminidase activity was in the linear region of the activity curve (Mckimm-Breschkin *et al.,* 1996). The instability of the neuraminidase, as observed in these experiments, was thought to be due to the higher entropic level of the unfolded mutant enzyme in contrast to the properly folded wild-type neuraminidase. If this is correct, then the question arises as to why the mutant enzyme is able to fold at all in order to yield an active enzyme. It must be kept in mind that the 4-GuDANA-resistant virus was able to undergo multiple rounds of replication in eggs and in tissue culture and replicated to titers equal to or greater than those of wild-type virus (Staschke *et al.,* 1995). Additionally, when kept at room temperature overnight, the remaining activity of the mutant enzyme was as stable as that of the wild-type enzyme, indicating that the mutant enzyme had reached equilibrium (A. J. Baxter and J. M. Colacino, unpublished data).

In this report we determined enzyme kinetic parameters for both 4-GuDANA-resistant and wild-type N9 neuraminidase. Both N9 enzymes had equivalent K_m values for the MUN substrate but, as expected, the K_i for the inhibitor 4-GuDANA was much higher for the mutant enzyme. Additionally, both enzymes displayed a preference for the 2-3′ linked rather than the 2-6′ linked sialic acid substrate, as has been seen for neuraminidases from other strains of influenza virus (Nagai *et al.,* 1995; Corfield *et al.,* 1982; Drzeniek, 1967).

FIG. 5. The active site of 4-GuDANA-resistant N9 neuraminidase. A map of the electron density clearly indicates the absence of the Glu side chain for amino acid residue in position 119, which is now replaced by Gly. Also note the presence of the strong structural water molecule or anionic species taking the place of the carboxylate of Glu 119. The (2Fo-Fc)ac map was calculated at 2.07 Å resolution and contoured at the 1.0 σ level.

FIG. 6. Ribbon diagram of the wild-type N9 neuraminidase tetramer showing the locations of Glu119 and Arg156 (N2 numbering).


Gly119
Peak 1
Peak 2
5


119
156
119
156
156
119
156
119
6

We have now found, using electron microscopic analysis of wild-type and 4-GuDANA-resistant neuraminidase heads, that preparations of mutant enzyme consisted mainly of monomers and dimers with only a small percentage (<10%) of tetramers, in contrast to wild-type enzyme, which consisted of virtually all tetramers. Cross-linking studies, using the NHS-ester cross linker DTSSP, are consistent with these findings.

To our knowledge, no experiments that show whether influenza virus neuraminidase monomers are active have been reported. The monomers of the E119G mutant are clearly not active, but it is not known if monomers obtained by pronase treatment of virions retain the same structure as monomers present in the viral envelope and containing a stalk and anchor domain. However, the observation that the monoclonal antibody NC10, which is specific for monomers within the tetrameric enzyme, do not bind to E119G monomers with the same efficiency (Mckimm-Breschkin *et al.,* 1995) indicates that the structure of these monomers is different from those assembled into the tetramer. No crystals of influenza NA monomers have been obtained.

The question is raised as to how a single amino acid substitution of Glu119 to Gly results in the tetrameric neuraminidase head dissociating into monomers. Glu119 (N2 numbering), which is an amino acid conserved in all influenza virus neuraminidases studied to date, forms a salt link with Arg156 (N2 numbering), another conserved amino acid (see Fig. 4 in Staschke *et al.,* 1995). Arg156 is positioned at the intersubunit interface (Fig. 6) and elimination of the salt link between Arg156 and Glu119, as occurs when Glu119 is replaced with Gly, might destabilize the tetramer so that while it still forms during virus replication and is located in the viral envelope still attached to its stalk, it falls apart into monomers during isolation and purification of the enzyme heads. To address this question, CHARMM force field calculations were performed on the wild-type N9 neuraminidase and on the 4-GuDANA-resistant (E119G) enzyme to investigate the interaction energies of association of the monomers into a hypothetical tetramer. The results of the calculations indicated that the formation of the tetramers of the wild-type enzyme is energetically more favorable than the formation of tetramers of the E119G mutant enzyme. Arg156 in the wild-type enzyme was shown to have a −3.1 kcal/mol favorable energy of interaction with the surrounding enzyme environment in the wild-type enzyme. In contrast, Arg156 in the mutant enzyme had a 17.7 kcal/mol unfavorable energy of interaction with the surrounding environment. Taken together, these calculations indicate that E119G tetramers would tend to dissociate into monomers more readily than would wild-type tetramers and that the interaction of Arg156 with Glu119 plays a role in tetramer formation and tetramer stability. Since the C-4 guanidinium group of 4-GuDANA participates in a strong electrostatic interaction with the Glu119, the salt link between Glu119 and Arg156 might be displaced in the presence of the inhibitor. To this end, it would be of interest to determine whether 4-GuDANA can destabilize wild-type neuraminidase.

ACKNOWLEDGMENTS

We thank Garry Taylor (University of Bath, Bath, UK) and Karin Briner and Jeff Radding (Lilly Research Laboratories, Indianapolis, IN) for helpful discussions and Aulikki Koskinen (Australian National University, Canberra, Australia) for excellent technical assistance.

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